

DUAL-FUNCTION MICROBICIDES

5

Field of the Invention

The present invention is directed to cyclohexenyl-substituted thiourea compounds useful in providing contraceptive, e.g. spermicidal, effects. In one particular embodiment, the present invention is directed to novel dual-function cyclohexenyl-substituted thiourea compounds that exhibit both spermicidal activity and potent anti-
10 microbial, particularly anti-viral, activity.

Background of the Invention

Human immunodeficiency virus (HIV), the etiologic agent of acquired immunodeficiency syndrome (AIDS) is the fastest growing cause of death in women of
15 reproductive age [1-3]. Worldwide, heterosexual transmission accounts for 90% of all HIV infections in women [2, 4]. Currently an estimated 14.1 million women worldwide are infected with HIV, representing 44% of all adult infections. Considering that the AIDS pandemic is still in its infancy on a global scale, this evolving demographic situation warrants urgent attention particularly for the adolescent population. Therefore,
20 effective strategies are needed to reduce heterosexual and perinatal HIV transmission. In the absence of an effective prophylactic anti-HIV therapy or vaccine, new emphasis has been placed on the development of intravaginal microbicides capable of reducing the transmission of HIV [5, 6]. In addition, prophylactic contraception is fundamentally important in HIV-infected women for prevention of HIV transmission and pregnancy,
25 especially because 80% of women with AIDS are of childbearing age [7].

At present, all commercially available spermicidal microbicides have detergent ingredients that disrupt cell membranes [8, 9]. The most widely used vaginal spermicide, nonoxynol-9 (N-9), because of its membrane disruptive properties has been shown to damage the cervicovaginal epithelium [10-12], cause an acute inflammatory tissue
30 response [13], alter vaginal microflora [14, 15], and enhance the risk of promoting

opportunistic infections in the genitourinary tract [16]. Such opportunistic infections are known to enhance the susceptibility of the ectocervical epithelium and the endocervical mucosa to HIV infection [17-19]. Despite its ability to inactivate HIV *in vitro*, the reported failure of N-9 to prevent heterosexual vaginal transmission of HIV in clinical settings in addition to its adverse effects on the cervicovaginal epithelium and vaginal microflora has prompted the search for new female-controlled microbicides that are both more effective and safer than N-9 [20-24]. Unlike the detergent-based microbicides that target cell membranes, the intravaginal or intrarectal use of topical formulations of anti-HIV drugs such as non-nucleoside inhibitors (NNIs) might be an effective approach for preventing the sexual transmission of HIV. These inhibitors of viral replication have been proposed by the WHO as candidates for intravaginal microbicides to inhibit HIV replication in mucosal cells [25, 26]. Inasmuch as physiological fertilization is dependent on the ability of ejaculated sperm to swim, bind the zona pellucida, and penetrate the egg, that are primarily dependent on sperm motility, adding spermicidal function to potent anti-HIV drugs could be an effective way to curb heterosexual HIV transmission as well as prevent conception.

Design of potent inhibitors of HIV-1 reverse transcriptase (RT) has been a focal point in translational AIDS research [27-30]. The NNIs are a diverse set of compounds which include tetrahydroimidazobenzodiazepinethione (TIBO) compounds [31], 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives [32-35], bis(heteroaryl)piperazine (BHAP) analogs[30], 2'-5'-bis-O-(tertbutyldimethylsilyl)-3'-spiro-5''-(4''-amino-1'', 2''-oxathiole-2'', 2''-dioxide) pyrimidine (TSAO) [36], dihydroalkoxybenzyloxypyrimidine (DABO) [37, 38], and phenethylthiazolylthiourea (PETT) derivatives [39-42]. These NNIs interfere with the activity of viral RT by binding to a specific allosteric site of HIV-1 RT near the polymerase site and severely limit the conformational flexibility needed for RT function, rendering the viral protein inactive [43-49]. A number of crystal structures of RT complexed with NNIs have been reported, and such structural information has provided the basis for further derivatization of NNI aimed at maximizing binding affinity to RT [44-50].

The success of NNIs for the clinical treatment of AIDS has led us to the computer-aided design and chemical synthesis of mechanism-based anti-retroviral agents that also exhibit spermicidal activity. Recently, we described structure-based design and synthesis of novel NNIs by generating a novel computer model in which a composite binding pocket was constructed from 9 individual crystal structures of RT-NNIs complexes [51, 52]. This computer docking procedure revealed abundant sterically allowed usable space surrounding the pyridyl ring of the thiourea compound, trovirdine. Using this model, we strategically designed novel compounds having functional groups that better fit and interact in the binding pocket space, to obtain more potent anti-HIV agents with higher affinity for the NNI binding pocket of HIV-1 RT that also exhibit spermicidal activity [53]. Continued development of novel agents designed to utilize the spatial and chemical relationships defined in the RT-NNI binding pocket model is needed to obtain alternative potent anti-microbial agents, and preferably, dual-function microbicides exhibiting spermicidal activity.

Summary of the Invention

The present invention provides novel thiourea compounds that are useful as anti-microbial, e.g., anti-viral, and contraceptive agents, as well as products and methods using these compounds. Examples of such useful products include vaginal foams, creams, lotions or gels, sponges or other vaginal inserts, and condom lubricating compositions. The present invention also is directed to certain thiourea compounds that exhibit contraceptive properties while maintaining activity against microorganisms, such as HIV.

Particularly useful compounds of the invention are cyclohexenyl-substituted thioureas as described more fully below. Specific cyclohexenyl ring-containing thiourea compounds are identified in the Examples below as preferred, potent, dual-function anti-microbial and spermicidal agents. In particular, PHI-346 (N-[2-(5-bromopyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl]-thiourea); and PHI-445 (N-[2-(5-chloropyridinyl)]-N'-[2-

cyclohexenyl)ethyl-thiourea), were identified as most preferred dual-function anti-HIV spermicides.

The present invention also provides contraceptive products that utilize the dual function spermicidal thiourea compounds of the invention, preferably substituted cyclohexenyl thiourea compounds, as active agents, and to the production of such contraceptive products.

Methods of the invention include the step of contacting sperm with a spermicidal thiourea compound of the invention, for example by means of a contraceptive product of this invention as discussed above.

Brief Description of the Drawings

Figure 1 is a graph showing concentration-dependent inhibition of human sperm motility by cyclohexenyl ring-containing substituted thiourea derivatives. Highly motile fractions of sperm were incubated with increasing concentrations (15.6 μ M-1000 μ M) of PHI-346, PHI-347, and PHI-445 or 1% DMSO in the assay medium, and the percentage of motile sperm were evaluated by CASA. Each data point represents the mean from three independent experiments.

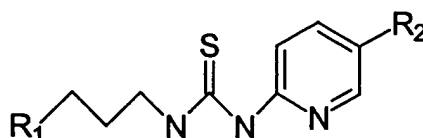
Figures 2A-2C are graphs showing the concentration-dependent effect of cyclohexenyl ring containing substituted thiourea derivatives on human sperm motion kinematics. Highly motile fraction of sperm were incubated with increasing concentrations of substituted cyclohexenyl thioureas or 1% DMSO in the assay medium, and their effects on, average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity were evaluated by CASA. Results are expressed as the mean \pm SD for 3 separate experiments. PHI-346 (A); PHI-347 (B); and PHI-445(C).

Detailed Description of the Invention

Compounds of the Invention

The compounds of the invention are thiourea compounds useful as potent anti-
5 microbial, particularly anti-HIV, agents as well as spermicidal agents, as demonstrated in the Examples below. The preferred agents exhibit reduced side effects when compared with N-9.

The compounds of the invention have the general structure shown below:



10 Preferred compounds are those where R_1 is cyclohexenyl, preferably 1-cyclohexenyl, and R_2 is CF_3 or halo, preferably bromo or chloro. Spermicidal compounds of the invention also include those where R_1 is pyridyl (e.g., PHI-207) and piperidiny (e.g., PHI-344).

15 Cyclohexenyl substituted thiourea derivatives, more particularly 1-cyclohexenyl ring-containing derivatives are preferred dual function agents. 5-bromo (PHI-346) and 5-chloro (PHI-445) functionalized cyclohexenyl ring-substituted thioureas are most preferred.

The heterocyclic and cyclohexenyl-substituted thiourea compounds of the invention, and particularly PHI-346 (N-[2-(5-bromopyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl-thiourea) and PHI-445 (N-[2-(5-chloropyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl-thiourea) are particularly useful as active ingredients of a vaginal contraceptive, for example, in women who are at high risk for acquiring HIV or other microbial infections by heterosexual vaginal transmission.

25 It will be readily apparent to those skilled in the art that other structures functioning as precursors and derivatives such as salts and esters of the present structures can be developed. The compounds of the invention can be prepared and characterized as described in the Examples below.

Contraceptive Compositions of the Invention

The compounds of the invention, as spermicidal active agents, can be formulated into contraceptive compositions for use. Such compositions are intended particularly for use with mammals, i.e. any class of higher vertebrates that nourish their young with milk secreted from mammary glands, for example humans, rabbits and monkeys. It is expected that the present invention will be used by humans, in most practical applications.

The contraceptive compositions of the present invention contain one or more of the spermicidal thiourea compounds. The total amount of spermicide will typically range from about 0.025 to 0.5 weight percent based on the total weight of the contraceptive composition. The amount of spermicide employed generally will be that amount necessary to achieve the desired spermicidal and anti-viral protective results. The amounts can be varied as needed for specific compositions. Preferably, the amount of the spermicidal thiourea compound employed will be from about 0.05 to 0.5 weight percent, and more preferably from about 0.05 to 0.25 weight percent, based on the total weight of the contraceptive composition.

The contraceptive compositions of the present invention contain not only the spermicidal thiourea compound of the invention, but also pharmaceutically acceptable carriers, diluents or vehicles as needed, i.e., materials for appropriately delivering and/or maintaining the spermicidal thiourea compound to a site for contact with sperm and so as to provide the desired spermicidal and anti-microbial, e.g., anti-viral protective activity.

One advantageous component in the pharmaceutical composition for administration of a spermicide is a polymeric delivery component as described in U.S. Patent 5,595,980, which patent is incorporated herein by reference. It has been found that such polymeric delivery component enhances the effectiveness of a spermicide and reduces vaginal irritation on administration.

In addition to the polymeric component, the balance of the contraceptive compositions, i.e., typically from about 0.1 to 99.8% and, often, about 50 to 99.8 weight percent, may optionally comprise one or more cosmetic ingredients. Such cosmetic

ingredients are known to those skilled in the art and are often referred to in the art as diluents, solvents, and adjuvants. Typically cosmetic ingredients include, for example; water, ethyl alcohol, isopropyl alcohol, glycerin, glycerol propylene glycol, sorbitol, and other high molecular weight alcohols. In addition, contraceptive compositions may
5 contain minor amounts, e.g. from about 0.1 to 5% weight based on the weight of the contraceptive compositions, of other additives, such as, for example; stabilizers, surfactants, menthol, eucalyptus oil, other essential oils, fragrances, and the like. Polyoxyethylene 20 sorbitan monolaurate is a preferred stabilizer for use in the compositions. The selection and amounts of cosmetic ingredients, other additives, and
10 blending procedures can be carried out in accordance with techniques well-known in the art.

The spermicidal active ingredients, and contraceptive compositions containing the same, of the present invention may be delivered to the vagina of a mammal by any means known to those skilled in the art. Typical forms for delivery of the compositions include,
15 for example; creams, lotions, gels, foams, intervaginal devices such as sponges and suppositories, and films. In addition, the contraceptive compounds and compositions of the present invention may be used as personal care products, such as, for example, condom lubricants, and the like. Such lubricants may comprise commonly known ingredients such as, for example: humectants; e.g., glycerin, sorbitol, mannitol, glycols
20 and glycol ethers; buffers, e.g., glucono-d-lactone; germicides or bactericides; e.g., chlorhexidine gluconate; preservatives, e.g., methylparaben; viscosifiers; e.g., hydroxyethyl cellulose, etc.; other adjuvants; e.g., colors and fragrances; in addition to the compositions of the present invention. Those skilled in the art will recognize that the physical properties, e.g., viscosity, of such delivery forms may vary widely. For
25 example, the viscosity of a gel form of the composition of the present invention, e.g., 150,000 centipoise, may be substantially higher than the viscosity of lotion form of the composition of the present invention, e.g., 100 centipoise. Further details concerning the materials, ingredients, proportions and procedures of such delivery forms can be selected in accordance with techniques well-known in the art.

30 The contraceptive compositions of the present invention are preferably

administered to the vagina of the mammal in a dosage which is effective to immobilize sperm present in the vagina and/or to inhibit their penetration in cervical mucus. Typical dosages range between about 0.0001 to 0.001 grams of the composition per kilogram of body weight of the mammal.

Intervaginal devices also may be used to aid in the administration of the
spermicidal active ingredients or contraceptive compositions containing the same as
described in U.S. Patent 5,069,906, the disclosure of which is incorporated herein by
reference.

In administering the spermical active ingredients in the form of the above
10 compositions, the compositions also may be formulated to release the spermicide both
rapidly and with a prolonged release of the drug. Such a formulation providing both
rapid and prolonged release has been described in U.S. Patent 4,707,362, which also is
incorporated herein by reference.

15 **EXAMPLES**

The invention will be explained further with reference to the following examples, which should not be considered to limit the invention.

Materials And Methods

Reagents and Instrumentation

All chemicals were purchased from Aldrich Chemical Corporation (Milwaukee, WI). Anhydrous acetonitrile and N,N-dimethylformamide were transferred to reaction vessels *via* cannula under nitrogen. All reactions were carried out under nitrogen. Proton (¹H), carbon (¹³C), and fluorine (¹⁹F) nuclear magnetic resonance spectra (NMR) were recorded on a Varian Oxford 300 MHz spectrometer (Varian Associates, Palo Alto, CA) using an automated broadband probe. All NMR spectra were recorded in CDCl₃. ¹³C NMR spectra were recorded using the proton decoupling technique. ¹⁹F NMR spectra were recorded in CDCl₃ and a 1% solution of trifluoroacetic acid in water was used as an internal standard in a fused capillary tube. Fourier transform infrared (FT-IR) spectra

were recorded using a FT-Nicolet Model Protege 460 instrument (Nicolet Instrument Corp., Madison, WI). Mass spectra analyses were performed by using a Model G2025A Hewlett Packard matrix-assisted laser desorption spectrometer (Wilmington, DE) in the molecular ion detection mode. The matrix used was cyano hydroxy cinnamic acid.

5 Ultraviolet (UV) spectra were recorded from Beckmann Model 3DU 7400 UV-Visible spectrophotometer (Beckmann Instruments, Fullerton, CA) using a cell path length of 1 cm. Elemental analysis was performed by MicroLab Inc., (Atlanta, GA). Column chromatography was performed using silica gel. The solvents used for elution varied depending on the compound and included one of the following: ethyl acetate, methanol,
10 chloroform, hexane, methylene chloride and ether.

Synthetic Scheme for Substituted Thiourea Derivatives

The names of the 31 thiourea compounds synthesized and tested in this study are listed in Table 1. Thirty (30) novel thiourea compounds were designed as inhibitors of
15 HIV-1 RT, based on a composite binding pocket computer model constructed from nine (9) individual crystal structures of RT-NNI complexes [51, 52]. Modeling studies for rational drug design included the construction of a composite NNI binding pocket for nine RT-NNI crystal structures, the analyses of surface complementarity between NNIs and RT, and application of inhibitory constants (K_i values) combined with a docking
20 procedure involving the novel thiourea compounds [51, 52]. This computational approach allowed the identification of several ligand derivatization sites for the generation of more potent dual-function thiourea compounds. Detailed analysis of trovirdine-binding [51], revealed multiple, specific sites which where larger functional groups could be incorporated in to the NNI. The composite binding pocket, the docked
25 trovirdine molecule showed abundant sterically allowed usable space surrounding the pyridyl ring, the ethyl linker, and near the 5'-bromo position. We hypothesized that addition of strategically designed functional groups would yield more potent anti-HIV agents with contraceptive acitivity (e.g., SIA). A series of thiourea compounds was synthesized, in which one of the nitrogen atoms of the thiourea was attached either to a
30 phenyl, heterocyclic, or alicyclic moiety through a ethyl bridge and the other nitrogen

atom was attached to a 5'-bromo or 5'-trifluoromethyl substituted or unsubstituted pyridyl ring.

TABLE 1. Thiourea compounds synthesized and tested

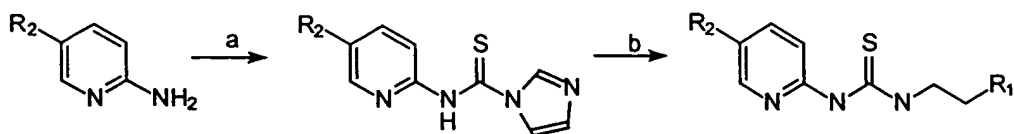
Compound	Chemical name
PHENYL	
HI-232	N-[2-(3,4-dimethoxyphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-236	N-[2-(2,5-dimethoxyphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-237	N-[2-(2-methoxyphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-239	N-[2-(3-methoxyphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-240	N-[2-(2-fluorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-241	N-[2-(3-fluorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-242	N-[2-(4-fluorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-243	N-[2-(4-bromophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-244	N-[2-(4-methylphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-253	N-[2-(2-chlorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-254	N-[2-(3-chlorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-255	N-[2-(4-chlorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-256	N-[2-(4-hydroxyphenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-275	N-[2-(phenethyl)]-N'-[2-(5-bromopyridinyl)]-thiourea
HI-308	N-[2-(phenethyl)]-N'-[2-(5-trifluoromethylpyridinyl)]-thiourea
HI-310	N-[2-(5-dimethoxyphenethyl)]-N'-[2-(5-trifluoromethylpyridinyl)]-thiourea
HI-311	N-[2-(2-fluorophenethyl)]-N'-[2-(5-trifluoromethylpyridinyl)]-thiourea
HI-345	N-[2-(3-fluorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HETEROCYCLIC	
HI-142	N-[2-(pyridinyl)]-N'-[2-(5-bromopyridinyl)]-thiourea (Trovirdine)
HI-172	N-[2-(1-piperidinoethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-258	N-[2-(1-piperizinyloethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-230	N-[2-(1-pyrrolidinylethyl)]-N'-[2-(5-bromopyridyl)]-thiourea
HI-207	N-[2-(2-pyridylethyl)]-N'-[2-(pyridyl)]-thiourea
HI-309	N-[2-(5-trifluoromethylpyridinyl)]-N'-[2-(pyridinyl)]-thiourea
HI-344	N-[2-(5-trifluoromethylpyridinyl)]-N'-[2-(1-piperidinyloethyl)]-thiourea
HI-443	N-[2-(5-bromopyridinyl)]-N'-[2-(2-thiophenylethyl)]-thiourea

ALICYCLIC

HI-346	N-[2-(5-bromopyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl]-thiourea
HI-347	N-[2-(5-trifluoromethylpyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl]-thiourea
HI-445	N-[2-(5-chloropyridinyl)]-N'-[2-(1-cyclohexenyl)ethyl]-thiourea
HI-444	N-[2-(5-bromopyridinyl)]-N'-[2-(2-myrtanyl)-thiourea
HI-541	N-[2-(2-cyclohexenyl)ethyl]-N'-[2-(pyridinyl)]-thiourea

For the synthesis of novel 5'-substituted pyridyl thiourea compounds, additional substitutions on the phenyl ring included either fluoro, chloro, bromo or methoxy groups at *ortho* (2,5), *meta* (3) or *para* (4) positions on the phenyl ring. The general synthetic scheme followed was detailed in previous reports [51, 52, 56, 57], and is shown below.

- 5 Trovirdine (N-[2-(pyridyl)]-N'-[2-5-bromopyridinyl]-thiourea), was synthesized according to a literature procedure [42].



a) 1,1-thiocarbonyl diimidazole, acetonitrile, RT, 12 hours

10 b) DMF, 100° C, 15 hours, $R_1\text{CH}_2\text{CH}_2\text{NH}_2$

Determination of the Partition Coefficients (P_o)

Partition of the test compounds between *n*-octanol and water as a measure of their lipophilicity was determined by taking the UV absorption of each compound in *n*-octanol, water-saturated *n*-octanol (1:1), and *n*-octanol-saturated water (1:1). The UV absorption maxima were determined by selecting a wavelength with the maximum absorption peak in the water-saturated octanol layer and the corresponding peak in the octanol-saturated water. Three absorption spectra were obtained for each compound. The partition coefficient (P_o) was calculated as the ratio of the compound concentration present in the lipid phase to the compound concentration present in the aqueous phase.

In Vitro Assays of Anti-HIV Activity

The HTLV_{III}B strain of HIV-1 was propagated in CCRF-CEM cells and the virus stocks obtained from cell-free supernatants of infected cells were titered using MT-2 cells [58, 59]. Cell-free supernatants were harvested and frozen in 1 ml aliquots at -70°C. Titration of the stock virus was performed using MT-2 cells and the cytopathic effect of the virus used in this study was typical of HIV-1 on MT-2 cells [58, 59].

For *in vitro* assays of the anti-HIV-1 activities of phenyl, heterocyclic and alicyclic ring substituted thiourea compounds, normal peripheral blood mononuclear cells (PBMCs) from HIV-1 negative donors were cultured for 72 hours in RPMI 1640 medium (Gibco-BRL, Grand Island, NY) with 20% (v/v) heat-inactivated fetal calf serum, 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2g/L NaHCO₃, 50 µg/ml gentamicin, and 4 µg/ml phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection of 0.1 during a 1 hour adsorption period at 37°C in a humidified 5% CO₂ atmosphere. Stock solutions (10 mM) of thiourea compounds were prepared in dimethylsulfoxide (DMSO). Cells were cultured for 7 days in 96-well microtiter plates (100 µl/well; 2 × 10⁶ cells/ml, triplicate wells) in the presence and absence of various concentrations (0.001 µM to 100 µM) of the thioureas. Cells from non-infected controls were handled in the same way except the virus was omitted from the preparation. Aliquots of culture supernatants were removed from the wells on the 7th day after infection for p24 antigen as previously described [59, 60]. The p24 enzyme immunoassay was the unmodified kinetic assay available commercially (Coulter Corporation/Immunotech, Inc., Westbrook, ME). The assay uses a murine monoclonal antibody (mAb) to the HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant sample binds [56-58]. The plates were read on a ELISA reader (Molecular Devices, Sunnyvale, CA) at 650 nm and p24 levels, expressed as ng/ml, were calculated against known standards supplied by Coulter/Immunotech, Inc. Percent viral inhibition was calculated by comparing the p24 values for the test substance-treated infected cells with the p24 values for untreated infected cells (i.e., virus controls).

Each of the thirty one thiourea compounds was tested for RT inhibitory activity against purified recombinant HIV-1 RT using the cell-free Quan-T-RT system (Amersham Corp., Arlington Heights, IL), which utilizes the scintillation proximity assay principle [61]. The anti-HIV activity was expressed as the IC₅₀ value, calculated from the dose-response curves, and defined as the drug concentration that decreases p24 antigen production in HIV-1 infected PBMCs or the HIV-1 rRT activity, by 50%.

Assays of Sperm Immobilizing Activity (SIA)

To evaluate the effect of each of the 31 thiourea compounds on human sperm motility and kinematics, highly motile fractions of sperm were prepared from donor semen (n = 8) by discontinuous (90-45%) gradient centrifugation using Enhance Plus medium (Conception Technologies, San Diego, CA) and the "swim-up" method [53, 62]. The swim-up method was used after density gradient centrifugation, washing, and resuspension of sperm in BWB-3% BSA. Pooled swim-up fraction was washed once prior to spermicidal assay.

All donor semen specimens were obtained after informed consent and in compliance with the guidelines of the Parker Hughes Institute Institutional Review Board. Pooled motile sperm ($\geq 10 \times 10^6/\text{ml}$) prepared from 3-6 donors were suspended in 1 ml of Biggers, Whitten, and Whittingham's medium (BWW) containing 25 mM HEPES (Irvine Scientific, Santa Ana, CA), and 0.3% BSA in the presence and absence of serial two-fold dilutions of test substance (500 μM to 15.6 μM) in 0.5% DMSO. The stock solutions of thioureas were prepared in DMSO (100 mM) and diluted in assay medium to yield the desired concentrations (up to 1000 μM). Corresponding volume of DMSO (either 0.5% or 1%) was added to control tubes. After 3 hours of incubation at 37°C, the percentage of motile sperm was evaluated by CASA [53, 62-64]. The percent motilities were compared with sham-treated control suspensions of motile sperm. The SIA of the compounds was expressed as the mean EC_{50} values (the final concentration of the compound in medium that decreases the proportion of motile sperm by 50%) calculated from three independent experiments.

Sperm Kinematic Parameters

For CASA, 5- μl of each sperm suspension was loaded into a 20- μm Microcell slide (Conception Technologies) in a counting chamber at 37°C. At least 5-8 fields per slide were scanned for analysis using a Hamilton Thorne Integrated Visual Optical System (IVOS), version 10 instrument (Hamilton Thorne Research Inc., Danvers, MA). Each field was recorded for 30 seconds. The computer calibrations were set at 30 frames

at a frame rate of 30/second. Other settings were as follows: minimum contrast 8; minimum size 6; low-size gate, 1.0; high-size gate, 2.9; low-intensity gate, 0.6; high-intensity gate, 1.4; phase-contrast illumination; low path velocity at 10 $\mu\text{m/s}$, and threshold straightness at 80%; and magnification factor, 1.95.

5 The sperm kinematics parameters that were determined included numbers of motile (MOT) and progressively (PRG) motile sperm; curvilinear velocity (VCL; a measure of the total distance traveled by a given sperm during the acquisition divided by the time elapsed); average path velocity (VAP; the spatially averaged path that eliminates the wobble of the sperm head), straight line velocity (VSL; the straight-line distance from
10 beginning to end of track divided by time taken), beat cross frequency (BCF; frequency of sperm head crossing sperm average path), the amplitude of lateral head displacement (ALH; the mean width of sperm head oscillation), and the derivatives, straightness ($\text{STR} = \text{VSL}/\text{VAP} \times 100$) and linearity ($\text{LIN} = \text{VSL}/\text{VCL} \times 100$; departure of sperm track from a straight line). Data from each individual cell track were recorded and analyzed. At
15 least 200 motile sperm were analyzed for each aliquot sampled.

Assay for Cell Viability

The potential cytotoxicity of two phenyl ring- (PHI-240 and PHI-253), one heterocyclic-ring (PHI-207) and two cyclohexenyl (PHI-346 and 445) ring-containing
20 most potent dual-function thiourea compounds in comparison to N-9, against normal human vaginal, ectocervical, and endocervical epithelial cells (Clonetics Corporation, San Diego, CA) was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based assay [53, 65]. Briefly, exponential growing vaginal, ectocervical, and endocervical epithelial cells were seeded into 96-well plates at a density
25 of 2×10^4 cells/well and incubated for 24 h at 37°C prior to drug exposure. On the day of treatment, culture medium was aspirated from the wells and replaced with fresh medium containing serial two-fold drug concentrations ranging from 7.8 μM to 1000 μM . N-9 (IGEPAL CO-630; Rhone Poulenc, Cranbury, NJ) was diluted in PBS. Triplicate wells were used for each treatment. Culture plates were then incubated for 3 hours before

adding 10 μ l of MTT solution (5 mg/ml in PBS) to each well. Wells containing only medium and MTT were used as control for each plate. The tetrazolium/formazan reaction was allowed to proceed for 4 hours at 37°C, and then 100 μ l of the solubilization buffer (10% sodium dodecyl sulfate in 0.1% HCl) was added to all wells and mixed
5 thoroughly to dissolve the dark blue formazan crystals. After an overnight incubation at 37°C, the OD at 540 nm were measured using a 96-well multiscanner autoreader with the solubilization buffer serving as blank. To translate the OD₅₄₀ values into the number of live cells in each well, the OD₅₄₀ values were compared to those of standard OD₅₄₀-versus-cell number curves generated for each cell line. The percent survival was calculated
10 using the formula: % survival = Live cell number [test]/Live cell number [control] \times 100. The results were expressed as mean IC₅₀ values for three independent experiments. The IC₅₀ [MTT] was defined as the concentration required for 50% reduction in cell survival.

Statistical Analysis

15 Results are presented as the mean or mean \pm SD values from independent measurements. Non-linear regression analysis was used to find IC₅₀ and EC₅₀ values from the concentration-effect curves using the GraphPad PRISM version 2.0 software program (San Diego, CA).

Results

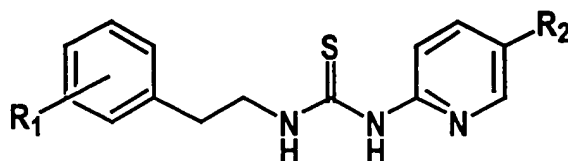
Anti-HIV and SIA of Phenyl Thiourea Compounds

Thiourea compounds were assayed for their ability to inhibit HIV-1 replication in normal human PBMCs infected with the HIV-1 strain HTLV_{III}B (reported as IC₅₀[p24]) as
25 well as for their RT inhibitory activity in cell-free assays using purified recombinant HIV-1 RT (reported as IC₅₀[rRT]). The SIA of thiourea compounds was evaluated by computer-assisted sperm analysis (reported as EC₅₀). The lipophilicity of the compounds was measured by their partition coefficients between *n*-octanol and water (reported as P_o).

The effect of ring substitution and functionalization of the pyridyl moiety of the thiourea derivative, trovirdine was tested. Table 2 lists the anti-HIV and SIA profiles of 18 phenyl ring substituted thiourea compounds in which one of the nitrogen atoms of the thiourea is attached to one of 16 different substituted phenyl rings (R_1) through an ethyl
5 bridge and the other nitrogen atom is attached to a 5'-bromo or 5'-trifluoromethyl substituted (R_2) pyridyl ring. Among the 16 substituted phenyl thiourea derivatives, functionalization of the phenyl ring with 2,5-dimethoxy (PHI-236), 2-fluoro (PHI-240), 3-fluoro (PHI-241), and 2-chloro (PHI-253) was associated with enhanced anti-HIV activity ($IC_{50[p24]} = < 1$ nM; $IC_{50[rRT]} = 0.10$ μ M to 0.70 μ M) when compared with the
10 unsubstituted phenyl thiourea compound (PHI-275) ($IC_{50[p24]} = 4$ nM and $IC_{50[rRT]} = 1.25$ μ M). In addition, phenyl ring-containing thiourea compounds substituted with 3-methoxy (PHI-239), 4-fluoro (PHI-242), and 4-chloro (PHI-255) functional groups were also potent inhibitors of HIV-1 ($IC_{50[p24]} = 1$ nM to 10 nM; $IC_{50[rRT]} = 0.4$ μ M to 6.4 μ M). By comparison, the unsubstituted 5'-trifluoromethyl phenyl thiourea (PHI-308) was
15 inactive and its alkoxy (PHI-310) and halo (PHI-311) substituted derivatives were less potent ($IC_{50[p24]}$ values of 0.08 μ M and 0.09 μ M and $IC_{50[rRT]}$ values of 2 μ M and 0.6 μ M, respectively. Thus, the substitution of the phenyl ring with various functional groups had a major impact on the anti-HIV activity of unsubstituted parent compound (PHI-275). Specifically, functionalization of 5-bromo phenyl substituted thiourea derivatives with
20 alkoxy or halo substitutions on the phenyl ring led to superior anti-HIV activity.

Exposure of the highly motile fraction of human sperm to 12 of the 18 substituted phenyl thiourea derivatives listed in Table 2 did not affect sperm motility even at concentrations as high as 500 μ M. Notably, halo-functionalized phenyl-substituted thiourea derivatives with 2-fluoro (PHI-240), 2-chloro (PHI-253), and 4-chloro (PHI-255)
25 functional groups showed, in addition to their potent anti-HIV activity ($IC_{50[p24]} = < 1$ nM), also spermicidal activity with EC_{50} values of 147 μ M, 70 μ M, and 160 μ M, respectively (Table 2). Thus, halo substitution of the phenyl ring was essential for the SIA of phenyl thiourea compounds.

TABLE 2. Effect of 18 phenyl ring substituted thiourea compounds on p24 antigen production in HIV-infected PBMC, enzymatic activity of HIV-1 rRT, and human sperm motility.



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Compound	R ₁	R ₂	IC ₅₀ [p24] ^a (μM)	IC ₅₀ [rRT] ^b (μM)	EC ₅₀ [SIA] ^c (μM)	P _a ^d
HI 232	3,4-dimethoxy	Br	ND ^e	>100	>500	22.7
HI 236	2,5-dimethoxy	Br	<0.001	0.10	>500	1.78
HI 237	2-methoxy	Br	0.01	1.0	>500	3.79
HI 239	3-methoxy	Br	0.003	0.40	>500	1.07
HI 240	2-fluoro	Br	<0.001	0.60	147 ± 18	1.20
HI 241	3 fluoro	Br	<0.001	0.70	>500	ND
HI 242	4-fluoro	Br	0.009	6.40	>500	10.7
HI 243	4-bromo	Br	0.07	0.90	>500	4.16
HI 244	4-methyl	Br	0.03	0.70	>500	1.49
HI 256	4-hydroxy	Br	3.06	87.7	233 ± 68	2.93
HI 253	2-chloro	Br	<0.001	0.70	70 ± 8	1.04
HI 254	3-chloro	Br	0.01	3.10	>500	6.20
HI 255	4-chloro	Br	0.001	2.50	160 ± 16	2.23
HI 275	H	Br	0.004	1.25	361 ± 31	3.50
HI 308	H	CF ₃	ND	>100	>500	ND
HI 310	2,5-dimethoxy	CF ₃	0.08	2.00	276 ± 22	1.00
HI 311	2-fluoro	CF ₃	0.09	0.60	>500	5.13
HI 345	3-fluoro	CF ₃	ND	0.70	>500	1.51

^aIC₅₀ [p24], Drug concentration inhibiting HIV-p24 antigen production by 50%.

^bIC₅₀ [rRT], Drug concentration inhibiting HIV-rRT activity by 50%.

^cEC₅₀[SIA], Drug concentration inhibiting sperm motility by 50%.

^dP_a, Partition coefficient. ^eND, not determined.

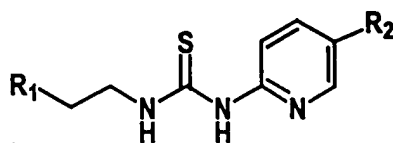
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The SIA of these dual-function thiourea derivatives was not related to their partition coefficient values (Table 2). The spermicidal compounds PHI-240, PHI-253, PHI-255, and PHI-275 had low P_a values (range 1.04 to 3.50). The lack of a correlation
5 between the P_a values and the biological activity of phenyl substituted thiourea derivatives demonstrates that these properties are not dependent on the lipophilicity of the compounds.

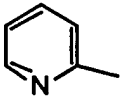

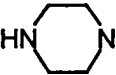
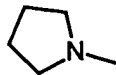
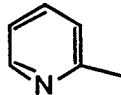
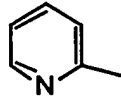
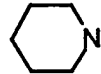
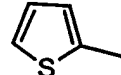
Anti-HIV and SIA of Heterocyclic Thiourea Compounds

10 Next, we synthesized a series of thiourea derivatives in which one of the nitrogen atoms of the thiourea is attached to one of 5 different heterocyclic substituents (R_1) through an ethyl bridge and the other nitrogen atom is attached to a 5-bromo or 5-trifluoromethyl substituted pyridyl ring. Table 3 lists the anti-HIV and SIA profiles
5 heterocyclic ring-substituted thiourea derivatives with and without 5-bromo or 5-trifluoromethyl functionalization (R_2) on the pyridine ring. When compared with trovirdine (listed as PHI-142), both the piperidinyl (PHI-172) and piperazinyl (PHI-258)-containing thiourea derivatives were more potent in inhibiting the replication of HIV-1 strain HTLV_{IIIB} in human PBMC with IC_{50} values of <1 nM and 2 nM, respectively, but they lacked SIA (EC_{50} values = > 500 μ M). Among the 8 heterocyclic thiourea
15 derivatives tested, the 5-bromo functionalized thioureas (PHI-172, PHI-258 and trovirdine) were more potent than trifluoromethyl substituted (PHI-309) or unsubstituted thioureas (PHI-207). Unlike trovirdine, which lacked SIA, the unsubstituted pyridyl thiourea (PHI-207) exhibited anti-HIV activity ($IC_{50[p24]} = 0.27 \mu$ M) as well as SIA ($EC_{50} = 279 \mu$ M). In addition, the more lipophilic piperidinyl derivative of trifluoromethyl
20 functionalized thiourea (PHI-344) exhibited SIA ($EC_{50} = 131 \mu$ M), but it lacked anti-HIV
25 activity ($IC_{50[RT]} = >100 \mu$ M) (Table 3).

TABLE 3. Effect of 8 heterocyclic ring substituted thiourea compounds on p24 antigen production in HIV-infected PBMC, enzymatic activity of HIV-1 rRT, and human sperm motility.



5

Compound		R ₁	R ₂	IC ₅₀ [p24] ^a (μM)	IC ₅₀ [rRT] ^b (μM)	EC ₅₀ [SIA] ^c (μM)	P _a ^d
Trovirdin ^e		(Pyridyl)	Br	0.007	0.8	>500	5.3
HI 172		(Piperidinyl)	Br	<0.001	5.8	>500	8.91
HI 258		(Piperazinyl)	Br	0.002	>100	>500	5.92
HI 230		(Pyrrolidinyl)	Br	1.0	>100	>500	7.91
HI 207		(Pyridyl)	H	0.27	0.39	279 ± 43	2.32
HI 309		(Pyridyl)	CF ₃	ND ^e	13.9	>500	3.81
HI 344		(Piperidinyl)	CF ₃	ND	>100	131 ± 5	10.27
HI 443		(Thiophene)	Br	0.03	5.3	>500	ND

^aIC₅₀ [p24], Drug concentration inhibiting HIV-p24 antigen production by 50%.

^bIC₅₀ [rRT], Drug concentration inhibiting HIV-rRT activity by 50%.

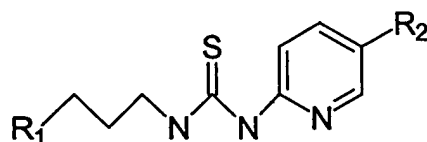
^cEC₅₀[SIA], Drug concentration inhibiting sperm motility by 50%.

10 ^dP_a, Partition coefficient. ^eND, not determined.

Anti-HIV and SIA of Alicyclic Thiourea Compounds

In addition to the compounds described above bearing phenyl and heterocyclic rings with and without 5'-functionalization, we also examined the effect of alicyclic ring-containing thiourea compounds. Table 4 lists the anti-HIV and SIA profiles of 5 alicyclic-substituted thiourea derivatives. Replacement of the pyridyl ring of trovirdine with a cis-myrtanyl (PHI-444) resulted in loss of anti-HIV activity, whereas replacement with a cyclohexenyl ring did not. The unsubstituted cyclohexenyl ring-containing inhibitor (PHI-541) lacked SIA ($EC_{50} = >500 \mu M$). However, functionalization at the 5'-position of the pyridyl ring of cyclohexenyl ring-containing thioureas with a bromine or a chlorine atom led to a significant increase in anti-HIV activity as well as gain of spermicidal function.

TABLE 4. Effect of 4 alicyclic ring substituted PETT derivatives on p24 antigen production in HIV-infected PBMC, enzymatic activity of HIV-1 rRT, and human sperm motility.



Compound	R ₁	R ₂	IC ₅₀ [p24] ^a (?M)	IC ₅₀ [rRT] ^b (?M)	EC ₅₀ [SIA] ^c ? (?M)	P _a ^d
Trovirdine	Pyridyl	Br	0.007	0.8	>500	5.3
HI 444	Cis-Myrtanyl	Br	ND ^e	>100	>500	2.1
HI-541	Cyclohexenyl	H	0.010	5.3	>500	ND
HI 346	Cyclohexenyl	Br	0.003	0.6	42 ± 9	8.6
HI 445	Cyclohexenyl	Cl	0.003	0.5	57 ± 5	6.3
HI 347	Cyclohexenyl	CF ₃	0.079	4.0	131 ± 4	11.5

^aIC₅₀ [p24], Drug concentration inhibiting HIV-p24 antigen production by 50%.

^bIC₅₀ [rRT], Drug concentration inhibiting HIV-rRT activity by 50%.

^cEC₅₀[SIA], Drug concentration inhibiting sperm motility by 50%.

^dP_a, Partition coefficient.

^eND, not determined.

Cyclohexenyl ring substituted thiourea derivatives with either 5-bromo (PHI-346) or 5-chloro (PHI-445) functionalization were the most potent dual-function agents with $IC_{50[p24]}$ values of 3 nM and EC_{50} values of 42 μ M and 57 μ M, respectively. Both of these compounds also inhibited HIV-1 RT activity with IC_{50} values of 0.6 μ M and 0.5 μ M, respectively. By comparison, the anti-HIV activity of the trifluoromethyl substituted cyclohexenyl thiourea derivative (PHI-347) was 26-fold less active and its SIA two to three-fold less active than those of halo functionalized thiourea derivatives. Thus, the potency order for SIA of cyclohexenyl substituted thiourea derivatives was bromo > chloro > trifluoromethyl. Spermicidal cyclohexenyl ring-containing thiourea derivatives had higher P_a values (6.3-11.5) than the inactive cis-myrtanyl substituted (2.1) thiourea derivative (PHI-444), suggesting that the biological activity of cyclohexenyl thiourea derivatives may in part be related to their increased lipophilicity.

All three cyclohexenyl ring-containing thioureas with additional substitutions on the 5'-position of the pyridyl ring induced a concentration-dependent SIA (Figure 1). The effective 100% inhibitory concentration (EC_{100}) was obtained at 0.25 mM for compounds PHI-346 and PHI-347, and at 1 mM for PHI-445. The concentration-dependent sperm motility loss induced by cyclohexenyl-substituted thiourea derivatives was also associated with significant changes in the movement characteristics of the surviving sperm particularly with respect to track speed (VCL), path velocity (VAP), and straight line velocity (VSL). The representative sperm kinematic parameters of cyclohexenyl-substituted thiourea-treated sperm *versus* concentration is shown in Figures 2A-2C.

As shown in the Figures, the decreases in VSL and VCL or VSL and VAP were similar in magnitude. Therefore, values for linearity (LIN) of the sperm tracks and the straightness (STR) of the swimming pattern remained relatively constant. Also, the beat cross frequency (BCF), and the amplitude of lateral sperm-head displacement (ALH) were relatively stable as the proportion of motile sperm declined during the linear phase of motility loss. The sperm motion parameters of control sperm showed no significant changes (data not shown).

Selective Spermicidal Activity of Dual-Function Thiourea Compounds

In order to determine whether the observed SIA was due to nonspecific membrane damaging effect of dual-function thiourea derivatives, the potential cytotoxicity of five most potent dual-function thiourea derivatives *versus* N-9 was tested against confluent monolayers of normal human vaginal, ectocervical, and endocervical epithelial cells using the MTT assay. Cells were exposed to these compounds at concentrations ranging from 7.8 μM to 1000 μM for 3 hours. The concentration-dependent cell survival curves for dual-function thiourea derivatives *versus* N-9 for these cells measured by the MTT assay were compared with the SIA measured by CASA (Table 5). In MTT assays, N-9 exhibited significant cytotoxicity to normal human vaginal, ectocervical and endocervical epithelial cells at spermicidal concentrations ($\text{EC}_{50} = 81 \mu\text{M}$) with mean $\text{IC}_{50[\text{MTT}]}$ values of 64 μM (SI = 0.7), 58 μM (SI = 0.7), and 32 μM (SI = 0.3), respectively. By comparison, the $\text{IC}_{50[\text{MTT}]}$ values for the most potent spermicidal dual-function thiourea derivatives against normal human vaginal, ectocervical, and endocervical epithelial cells were well above their spermicidal EC_{50} values (SI range = >6.8 to >23.8). Thus, unlike N-9 which was spermicidal only at cytotoxic concentrations, dual-function spermicidal thiourea derivatives showed high selectivity indices against these cells. Thus, spermicidal dual-function thiourea derivatives were significantly less active against reproductive tract epithelial cells.

TABLE 5. Effect of spermicidal thiourea compounds on the cell viability of normal human vaginal, ectocervical and endocervical epithelial cells as quantitated by MTT assay.

Compound	HIV	Sperm	Vaginal epithelial cells ^a		Ectocervical epithelial cells ^a		Endocervical epithelial cells ^a	
	IC _{50(p24)} (μ M) ^b	EC _{50(SIA)} (μ M) ^c	IC _{50(MTT)} (μ M) ^d	SI ^e	IC _{50(MTT)} (μ M)	SI	IC _{50(MTT)} (μ M)	SI
HI-207	0.27	279	>1000	>3.5	>1000	>3.5	>1000	>3.5
HI-240	<0.001	147	>1000	>6.8	>1000	>6.8	>1000	>6.8
HI-253	<0.001	70	>1000	>14.2	>1000	>14.2	>1000	>14.2
HI-346	0.003	42	>1000	>23.8	765 \pm 400	18.2	580 \pm 48	13.8
HI-445	0.003	57	578 \pm 25	10.1	692 \pm 139	12.1	185 \pm 21	3.2
N-9	NA ^f	81	64 \pm 3	0.7	58 \pm 1	0.7	32 \pm 1	0.4

5

^aConfluent monolayer cultures of normal human vaginal, ectocervical, and endocervical epithelial cells in 96-well plates were incubated in the absence and presence of increasing two-fold concentrations (7.8 μ M to 1000 μ M) of 5 potent dual-function PETT derivatives for 3 h at 37°C. Cell viability was measured by the MTT assay.

10 ^bIC_{50(p24)} = Drug concentration inhibiting HIV-p24 antigen production by 50%.

^cEC₅₀ = Drug concentration inhibiting sperm motility by 50%.

^dIC_{50(MTT)} = Drug concentration inhibiting cell growth by 50%; data are mean \pm SD of three experiments.

^eSI = Selectivity Index is equal to the ratio of IC₅₀ to EC₅₀.

^fNA = Not applicable.

15

While a detailed description of the present invention has been provided above, the invention is not limited thereto. This description contains numerous literature and patent citations, each of which is hereby incorporated by reference for all purposes, as if fully set forth. The invention described herein may be modified to include alternative embodiments, as will be apparent to those skilled in the art. All such alternatives should be considered within the spirit and scope of the invention, as claimed below.

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